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Kinetics of Hallmark Biochemical Changes in Paclitaxel-Induced Apoptosis

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ABSTRACT Apoptosis is associated with cascades of biochemical changes, including caspase activation, cleavage of poly-ADP-ribose polymerase (PARP), and fragmentation of genomic DNA. Knowledge of the kinetics of these changes in drug-induced apoptosis is important for designing pharmacodynamic studies. We have shown that the slow manifestation of apoptosis contributes to the delayed pharmacological effects of paclitaxel (Cancer Res. 58:2141-2148, 1998). The present study examined the timing of the biochemical changes in paclitaxel-induced apoptosis in human prostate PC3 cancer cells. After treatment with 20 nM paclitaxel, the fraction of cells that detached from the culture flask increased with time to reach 68% at the end of the 96-hour experiment. In contrast, the control samples showed <1% detachment. The attached and detached paclitaxel-treated cells showed different biochemical properties. The detached cells exhibited the full spectrum of apoptotic changes, whereas the attached cells only showed activation of caspase-3-like proteases but not PARP cleavage, DNA fragmentation, nor release of DNA fragments to the cytoplasm. Activation of caspases in the attached cells was several-fold lower and occurred at a later time (ie, 24 vs 12 hours) compared to the detached cells. In the detached cells, caspase activation was first detected at 12 hours and peaked at 36 hours, whereas PARP cleavage was first detected at 24 hours and was completed prior to 72 hours. In contrast, the extent of internucleosomal DNA fragmentation and the release of DNA-histone complex to the cytoplasm (both were first detected at 24 hours) were cumulative over time up to the last time point of 96 hours. In summary, in paclitaxel-induced apoptosis, caspase activation was followed with a 12-hour lag time by PARP cleavage, internucleosomal DNA fragmentation, and release of DNA-histone complex to the cytoplasm. There was no detectable lag time between PARP cleavage and DNA fragmentation. The observation that only the detached cells but not the attached cells showed the full spectrum of apoptotic changes suggests that detachment is either a part of the initiation/execution phases of apoptosis and/or is required for their completion.

KEY WORDS: paclitaxel, apoptosis, anoikis, caspase activation, PARP cleavage, DNA fragmentation

INTRODUCTION

Paclitaxel is one of the most important anticancer drugs developed in the last two decades. It has significant clinical activity against a variety of solid tumors and acute leukemia (1). Paclitaxel binds to and stabilizes microtubules, and induces mitotic arrest and apoptosis (2,3). Apoptosis is considered an important pharmacological action of paclitaxel.

Apoptosis is associated with morphological changes, including membrane blebbing, cellular shrinkage, chromatin condensation, and detachment from the extracellular matrix (4). It is also associated with biochemical changes, including activation of a cascade of proteases such as the caspases and endonucleases, cleavage of poly-ADP-ribose polymerase (PARP), and eventually fragmentation of genomic DNA (5-7). Caspase 3 is considered the first caspase involved in the execution phase of apoptosis and is activated by the proteins involved in the initiating phase (ie, caspase 8, caspase 9, and cytochrome C). Caspase 3 cleaves target proteins including PARP, gelsolin, p21-activated kinase 2, and DNA fragmentation factor (8-15). PARP is an essential DNA repair enzyme. Cleavage of PARP prevents DNA repair, activates a calcium/magnesium-dependent endonuclease, and results in internucleosomal DNA fragmentation. Cleavage of DNA fragmentation factor is also associated with internucleosomal DNA fragmentation. Internucleosomal DNA fragmentation, where the nuclear DNA is sequentially degraded to 300 kb, 50 kb, and ~185 bp fragments, is a late event in apoptosis that is frequently used to confirm apoptotic death (16). The fragmented DNA is released into the cytoplasm as a DNA-histone complex. Some of the above-mentioned biochemical changes have been observed in drug-induced apoptosis. For example, etoposide treatment results in activation of caspase 2 and caspase 3 in human leukemic U937 and HL-60 cells

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and in human epithelial MCF7 cells (17,18); lovastatin treatment results in activation of caspase 7 in human prostate LNCaP cells (19); and paclitaxel treatment results in PARP cleavage in HL-60 leukemic cells (20). In some cases, caspase activation was followed by PARP cleavage (17-19). Most of the published literature on apoptosis is focused on the mechanisms and regulation of apoptosis, the role of apoptosis in cancer etiology, and the induction of apoptosis by external stimuli including drugs. Relatively little is known about the kinetics of drug-induced apoptosis and there are no data on the timing of the hallmark apoptotic changes that occur during paclitaxel-induced apoptosis, separately and/or in relation to each other, in epithelial cancer cells. These kinetic data are needed for identifying the appropriate endpoints and the time points for measurements, and are therefore critical for studying the pharmacodynamics of drug-induced apoptosis. For example, the results of the present study showed that some apoptotic changes are transient with time. Measurements of transient changes should be made at the time when the changes occur and not after the changes have been completed. Conversely, inappropriate selection of apoptotic endpoints and/or time points will affect the pharmacodynamic data and thereby the data interpretation. We have previously shown that the lack of a thorough understanding of the kinetics of the apoptotic changes has confounded the design of studies that evaluated the pharmacodynamics of paclitaxel-induced apoptosis; measurement of its antitumor activity using different endpoints at different time points has led to conflicting conclusions on the effect of treatment duration on the drug effect (21). The objective of the present study was to delineate the sequencing and timing of the hallmark apoptotic changes in paclitaxel-induced apoptosis in epithelial cells, including caspase activation, PARP cleavage, and DNA fragmentation, and to identify the changes that provide quantitative data on the extent of apoptotic changes.

MATERIALS AND METHODS

Chemicals and Supplies

Paclitaxel was a gift from Bristol-Myers Squibb Inc. (Wallingford, Conn) and the National Cancer Institute (Bethesda, Md). ApoAlert CPP32 assay kit and ApoAlert Annexin V Apoptosis kit were purchased from Clontech Laboratories Inc (Palo Alto, Calif), C2.10 PARP monoclonal antibody from Enzyme Systems (Livermore, Calif), cell death detection ELISA kit from Boehringer Mannheim (Indianapolis, Ind), chemiluminescent Western blot kit from Amersham (Arlington Heights, Ill), RPMI 1640 and fetal calf serum from Life Technologies, Inc. (Grand Island, NY), cefotaxime sodium from Hoechst-Roussel (Somerville, NJ), gentamycin from Solo Pak Laboratories (Franklin Park, Ill), and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin from Dako Corp (Carpinteria, Calif). All chemicals and reagents were used as received.

Cell Culture

Human prostate PC3 tumor cells were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained as monolayer cultures in RPMI 1640 containing 10% heat inactivated fetal calf serum, 0.1 mM non-essential amino acids 100 mg/ml gentamycin, and 95 mg/ml cefotaxime and grown in a humidified atmosphere at 37°C in 5% CO_2.

Drug Treatment and Cell Collection

A stock solution of paclitaxel was prepared in ethanol. Sufficient volume of stock solution was added to the culture medium so that the final ethanol concentration was <0.1%. Cells (0.5×10^5/flask) were seeded in T-150 flasks and allowed to attach to the flask by growing in a drug-free medium for 48 hours. The media were then replaced with media containing paclitaxel. At predetermined times, the supernatant containing the detached cells was collected, followed by collection of the attached cells using trypsinization or by scraping with a rubber policeman. The cell number was determined using a Coulter counter. Afterwards, cells were centrifuged at 250× g and washed with phosphate-buffered saline (PBS) for 5 minutes.

Caspase Activity

The activity of Caspase 3 was measured using the ApoAlert CPP32 assay (Clontech Laboratories, Palo Alto, Calif). Briefly, 1×10^6 cells were lyzed using the lysis buffer provided in the assay kit. The lysate was stored at -20°C and analyzed within 1 week.

Enzyme activity was detected by the cleavage of the substrate, Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) to AFC, which emits a yellow-green fluorescence at 505 nm. The increase in caspase activity is measured as the ratio of the fluorescence intensity of a paclitaxel-treated sample to that of control cells collected at the same time.

PARP Cleavage

PARP (116,000 d) undergoes proteolytic cleavage between Asp 216 and Gly 217, to yield a fragment containing the COOH-terminal catalytic domain (~85,000 d), and a fragment containing a truncated NH 2-terminal DNA-binding domain (~26,000 d). Asp 216 is the preferred cleavage site for caspase-3 and other closely related proteases (11,22,23). PARP cleavage was analyzed by Western blotting. Briefly, 10^6 cells were washed with ice-cold PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 0.5 mg/ml each of leupeptin and apoprotinin), and resuspended in a reducing loading buffer (62.5 mM Tris, pH 6.8; 6 M urea; 10% glycerol; 2% sodium dodecyl sulfate (SDS); 0.003% bromophenol blue; 5% 2-mercaptoethanol). Protease inhibitors were used to minimize proteolytic cleavage of PARP during processing. The 2-mercaptoethanol solution was added (50 µl of 14.3 mmol/ml loading buffer) immediately prior to use. Samples were sonicated on ice, resuspended using a 21 gauge needle, and then incubated at 75°C for 15 minutes. After centrifugation, an aliquot representing 1.5 x 10^5 cells was loaded on a 10% SDS polyacrylamide gel and run at 30 mA overnight. The gel was transferred onto a 0.2 mm nitrocellulose membrane by electroblotting. The membrane was incubated with blocking solution (5% non-fat dry milk in PBS containing 0.1% Tween 20) for 1 hour, followed by incubation with the PARP monoclonal antibody overnight at 4°C and subsequently the anti-mouse immunoglobulin. After washing the membrane in PBS twice for 5 minutes each and once for 30 minutes, the immunoreactive bands were visualized by incubation of the membrane with the chemiluminescence Western blot kit.

Release of DNA-Histone Complex to Cytoplasm

The level of DNA-histone mono- and oligonucleosomes in the cytoplasm was measured using the cell death detection ELISA kit. Briefly, 5000 cells were lysed in the lysis buffer. The cytoplasmic fractions of the lysates were placed in a flask precoated with mouse antihistone primary antibody and mouse anti-DNA antibody conjugated to peroxidase. The peroxidase substrate, 2,2'-azido-di-[3-ethylbenzthiazoline sulfonate], was applied and the absorbance at 405 nm was measured.

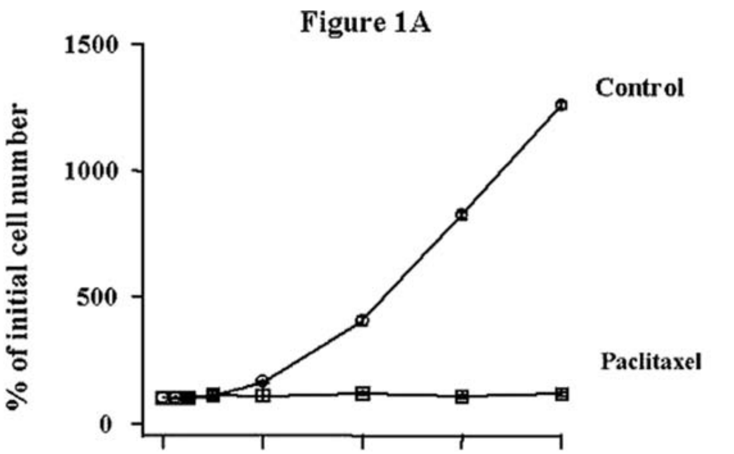
DNA Fragmentation

DNA fragmentation was measured by agarose gel electrophoresis using previously reported procedures (24). Briefly, cells were incubated at 37°C for 30 minutes in 10 mM Tris-HCl, 100 mM EDTA (pH 8.0), 20 mg/ml RNase A, and 0.5% SDS. The cell suspension was then treated with 200 mg/ml proteinase K at 50°C for 16 hours. DNA was extracted twice with phenol/chloroform (1:1) and once with chloroform, and precipitated by adding 0.2 volume 10 M NH 4Cl and 2 volumes ethanol. The pellet obtained after centrifugation was resuspended in 100 mM Tris-HCl and 10 mM EDTA (pH 8.0). The amount of DNA was measured by the absorbance at 260 nm, using a spectrophotometer. Samples showing a 260:280 absorbance ratio of >1.8, which ascertained the purity of the isolated DNA, were analyzed by gel electrophoresis. Equal amounts of DNA were loaded on a 1.5% agarose gel containing 0.5 mg/ml ethidium bromide, and run at 2 V/cm for 4-5 hours in 1× Tris-acetate/EDTA electrophoresis buffer. The DNA laddering pattern was visualized by UV transillumination and photographed.

RESULTS

Effect of Paclitaxel on Cell Growth and Detachment

Figure 1A shows the changes in cell number over time. In the absence of paclitaxel, the total number (attached plus detached) of PC3 cells increased by 12.6-fold after 96-hour incubation. In the presence of 20 nM paclitaxel, the total cell number remained constant, indicating growth retardation and/or cell kill by paclitaxel. Paclitaxel treatment further affected the detachment of cells from the extracellular matrix. Figure 1B shows the kinetics of cell detachment. Less than 1% of the untreated control cells were detached throughout the 96-hour experiment, whereas paclitaxel treatment induced cell detachment, increasing from 3% at 3 hours to 68% at 96 hours. The attached and detached paclitaxel-treated cells and the attached control cells were analyzed for caspase 3 activation, PARP cleavage, and DNA fragmentation. The number of detached cells in the controls was 5- to 50-fold lower than the detection limits which precluded analysis.



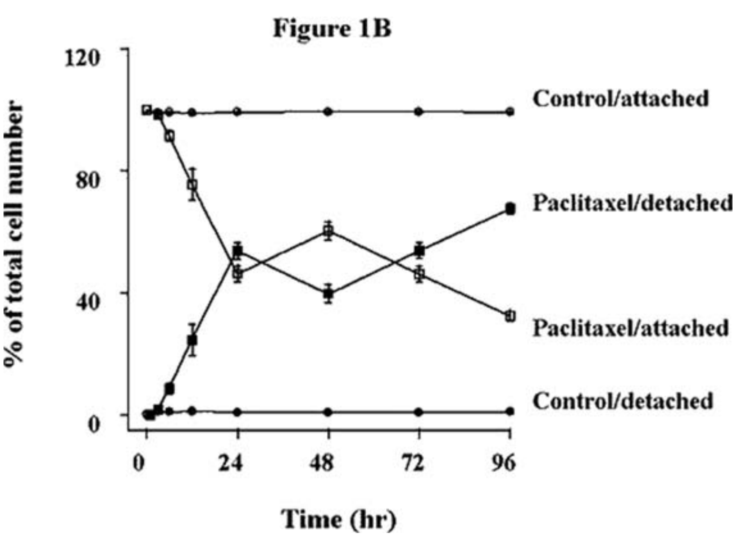


Figure 1. Time course of cell detachment. Panel A shows changes in total number (attached plus detached) of PC3 cells with time. Cells received continuous treatment with 0 nM (control, circles) and 20 nM paclitaxel (squares). Panel B shows kinetics of cell detachment. The numbers of cells that remained attached (open symbols) to the culture flask and the numbers of cells that were detached (solid symbols), with or without continuous treatment with 20 nM paclitaxel, were determined, and expressed as percentage of the total cell number. The average initial cell number was between 1 and 1.5 × 10^6. At the end of the 96-hour experiment, the average cell number was 1.5 × 10^7 for the untreated controls and between 1 and 1.5 & 10^6 for the paclitaxel-treated cells. Mean ± SD (n = 5).

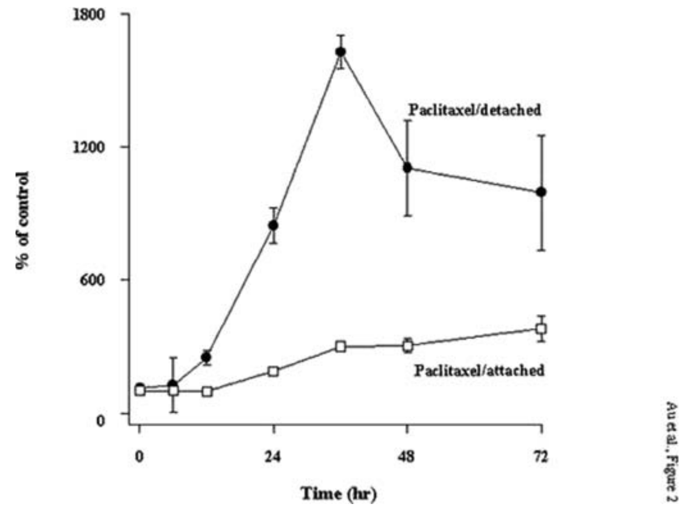


Figure 2. Activation of caspase-3-like proteases. The activity of caspase-3-like proteases was determined using the ApoAlert CPP32 assay. The caspase activity remained constant over time in the untreated controls. Caspase activity induced by continuous treatment with 20 nM paclitaxel is expressed as % of the control values. Mean ± SD (n = 6). Detached cells, solid symbols; attached cells; open symbols.

Caspase Activity

Figure 2 shows the caspase 3-like protease activity in PC3 cells. The activity in the attached cells remained constant in the absence of paclitaxel, whereas treatment with 20 nM paclitaxel resulted in enhanced enzyme activity. In the paclitaxel-treated cells that detached from extracellular matrix, caspase activity became significantly higher than the control value at 12 hours, reached a peak level that was about 16 times the control value at 36 hours, and subsequently declined with time to about 10 times the control value at 96 hours. The caspase activity in the paclitaxel-treated cells that remained attached was several-fold lower, with a maximum level of about 4 times the control value. Furthermore, the increase in caspase activity in attached cells started at a later time (ie, 24 hours) than in detached cells (ie, 12 hours).

PARP Cleavage

Figure 3 shows the Western blotting results. The attached cells in the untreated controls did not show PARP cleavage. For the paclitaxel-treated cells, only the detached cells but not the attached cells showed PARP cleavage. PARP cleavage in the detached cells began at 24 hours and was completed by 72 hours.

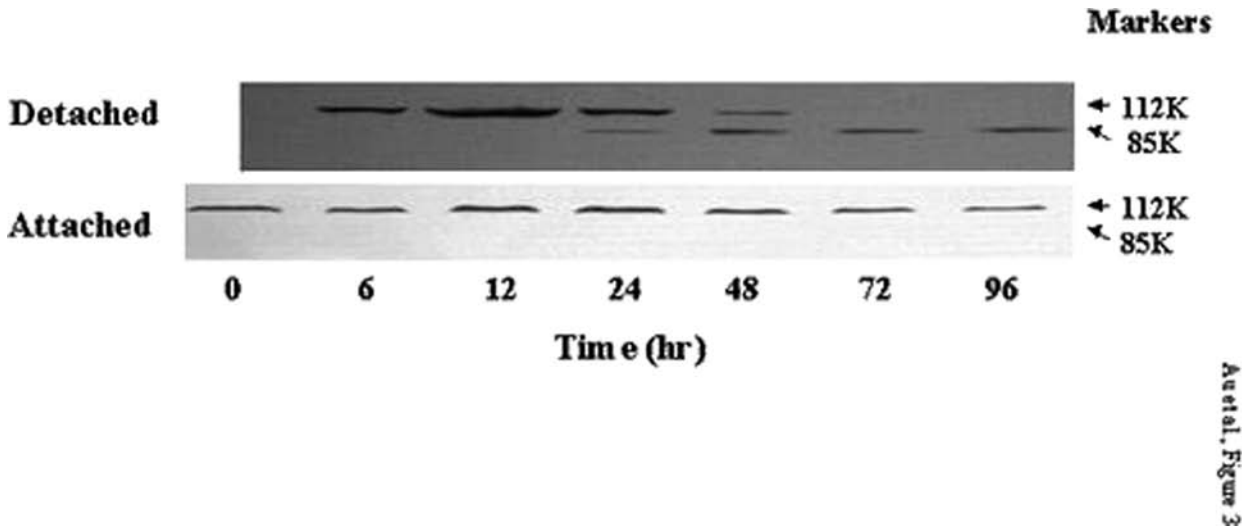


Figure 3. PARP cleavage. Cells were treated with 20 nM paclitaxel continuously. Cleavage of PARP (116,000 d) to its proteolytic product (85,000 d), as a function of time, was monitored using Western blotting. Molecular markers of 112,000 d and 85,000 d are noted on the right.

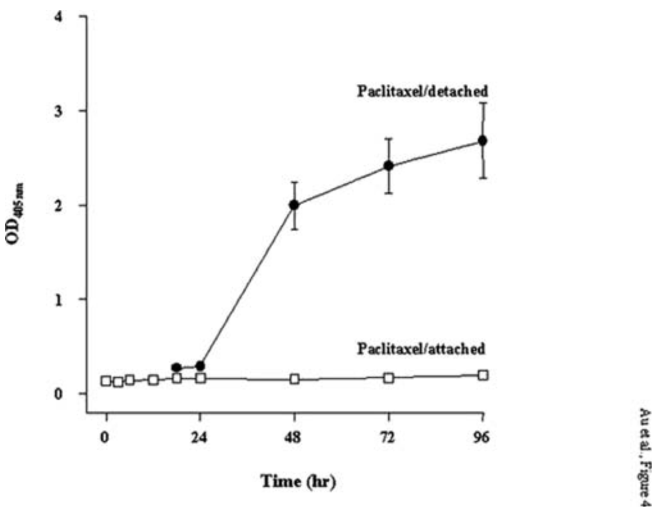


Figure 4. Cytoplasmic DNA-histone complex. Cells were treated with 20 nM paclitaxel continuously. Release of DNA-histone complex to the cytoplasm, as a function of time, was monitored by the optical density at 405 nm using ELISA (*n* = 3).

DNA Fragmentation

Figure 4 shows the results of the release of DNA-histone complex to the cytoplasm, and Figure 5 shows the DNA laddering. The attached cells in the untreated controls did not show DNA fragmentation by either method. For the paclitaxel-treated cells, the attached cells did not show measurable DNA fragmentation, whereas the detached cells showed cytoplasmic DNA-histone complex and DNA laddering at 24 hours, which continued to increase with time up to the last time point of 96 hours.

DISCUSSION

Results of the present study show that in human prostate carcinoma PC3 cells, treatment with paclitaxel resulted in detachment of cells from the culture flask. A comparison of the timing of the various biochemical changes in the paclitaxel-treated cells indicates that the sequence of the hallmark apoptotic changes in paclitaxel-induced apoptosis is in agreement with the current knowledge on the cascade of apoptotic changes, with caspase activation as the leading event that was first detected at 12 hours. The time for the detection of caspase activation in PC3 cells is identical to that in Jurkat T cells treated with micromolar concentrations of paclitaxel (25), suggesting similar kinetics in the caspase activation by paclitaxel in epithelial and T cells. Our data also indicate the lag time between the various steps in the apoptosis cascade. Caspase activation preceded the detection of PARP cleavage by12 hours, indicating a relative long lag time between the two changes. PARP cleavage was detected simultaneously with internucleosomal DNA fragmentation and the release of DNA-histone complex to the cytoplasm, suggesting a short or no lag time between PARP cleavage and DNA fragmentation.

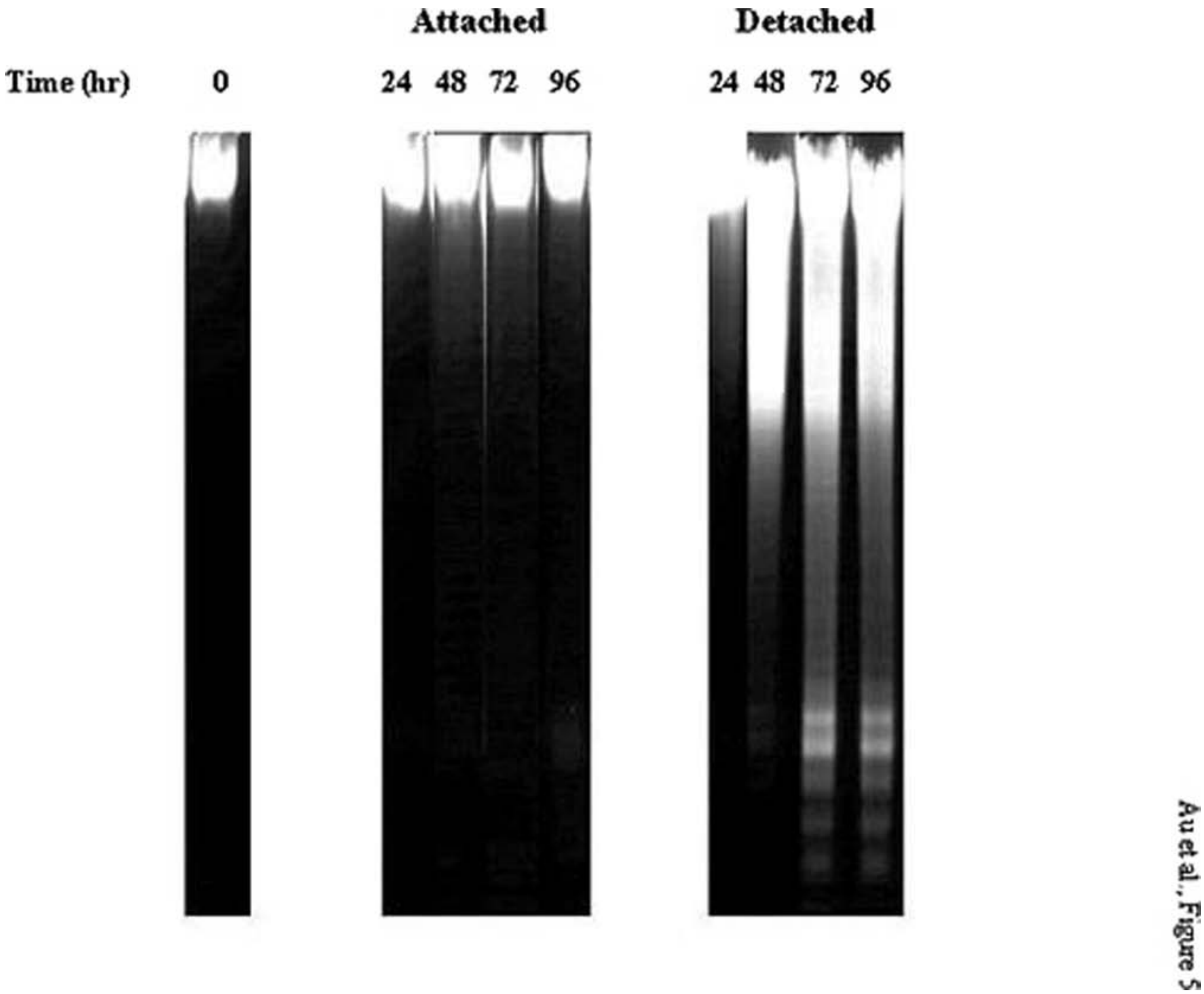


Figure 5. DNA fragmentation. Cells were treated with 20 nM paclitaxel continuously. DNA fragmentation, as a function of time, was monitored using gel electrophoresis.

The detached cells resulting from paclitaxel treatment showed the full spectrum of apoptotic changes, including caspase-3 activation, PARP cleavage, internucleosomal DNA fragmentation, and release of DNA-histone complex from the nucleus to the cytoplasm. In comparison, only caspase activation but not PARP cleavage nor DNA fragmentation was detected in the paclitaxel-treated attached cells. While the reasons for the differences in the biochemical properties of the paclitaxel-treated attached and detached cells are not apparent, the observation that the detached cells but not the attached cells showed the full spectrum of apoptotic changes suggests that detachment is either a part of the initiation/execution phases of apoptosis and/or is required for their completion. To our knowledge, this observation is the first to indicate an association between cell detachment and apoptosis due to paclitaxel treatment. Anoikis, a recently described phenomenon, is the process where apoptosis is induced by cell detachment from extracellular matrix due to disruption of the interaction between integrins in epithelial cells and extracellular matrix (26). In anoikis, epithelial cells, shortly after detaching from the extracellular matrix, activate Jun-N-terminal kinases and caspases (27). Cell death by anoikis is in agreement with our observation that detachment of cells from extracellular matrix preceded the detection of caspase activation and the subsequent apoptotic changes. However, it is noted that the inability of detecting caspase activation prior to detecting cell detachment may be the result of the limited assay sensitivity. It is also conceivable that the execution of paclitaxel-induced apoptosis involves other caspases that were not measured in the present study. It has been shown that in Jurkat T cells, treatment with micromolar concentration of paclitaxel results in two apoptotic pathways, including one that does not involve caspase activation (25). Further studies are needed to determine whether detachment occurs upstream of caspase activation and thereby determine the role of anoikis in paclitaxel-induced apoptosis.

In summary, our results indicate that in paclitaxel-induced apoptosis, caspase activation occurs relatively early, followed by PARP cleavage and DNA fragmentation. Pharmacodynamic studies using caspase activation as the endpoint of drug-induced apoptosis must take into account the significant lag time between caspase activation and disintegration of the genomic DNA. Furthermore, because caspase activation is not cumulative over time (whereas the number of cells undergoing apoptosis increases with time), the time course of caspase activation does not reflect the time course of appearance of apoptotic cells. Accordingly, caspase activation is not a good pharmacodynamic endpoint. On the other hand, the simultaneous detection of PARP cleavage and DNA fragmentation indicates that PARP cleavage can be used to determine the onset of DNA fragmentation (ie, DNA laddering and release of DNA-histone to the cytoplasm). Among these apoptotic changes, PARP cleavage and DNA laddering are measured by Western blotting and gel electrophoresis techniques, which are qualitative or semi-quantitative measurements, whereas the appearance of cytoplasmic DNA-histone complex is measured by ELISA and is therefore more quantitative. Based on the kinetics of the hallmark apoptotic changes, we recommend using the measurement of cytoplasmic DNA-histone complex at time points after 24 hours to study the pharmacodynamics of paclitaxel-induced apoptosis in epithelial cancer cells. In view of the significantly more extensive apoptotic changes in detached than in attached cells, pharmacodynamic evaluation of drug-induced apoptosis should consider separate measurements for the two cell populations.

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